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Abstract

Sleep and brain glutamatergic signaling are homeostatically regulated. Recovery sleep following prolonged wakefulness restores efficient functioning of the brain, possibly by keeping glutamatergic signaling in a homeostatic range. Evidence in humans and mice suggested that metabotropic glutamate receptors of subtype-5 (mGluR5) contribute to the brain's coping mechanisms with sleep deprivation. Here, proton magnetic resonance spectroscopy in 31 healthy men was used to quantify the levels of glutamate (Glu), GLX (glutamate-to-glutamine ratio) and GABA (γ-amino-butyric-acid) in basal ganglia (BG) and dorsolateral prefrontal cortex on 3 consecutive days, after ~ 8 (baseline), ~ 32 (sleep deprivation) and ~ 8 hours (recovery sleep) of wakefulness. Simultaneously, mGluR5 availability was quantified with the novel radioligand for positron emission tomography, [^{18}F]PSS232, and the blood levels of the mGluR5-regulated proteins, fragile-X mental retardation protein (FMRP) and brain-derived neurotrophic factor (BDNF) were determined. The data revealed that GLX ($p = 0.03$) in BG (for Glu: $p < 0.06$) and the serum concentration of FMRP ($p < 0.04$) were increased after sleep loss. Other brain metabolites (GABA, N-acetyl-aspartate, choline, glutathione) and serum BDNF levels were not altered by sleep deprivation ($p_{all} > 0.6$). By contrast, the night without sleep enhanced whole-brain, basal ganglia and parietal cortex mGluR5 availability which was normalized by recovery sleep ($p_{all} < 0.05$). The findings provide convergent multimodal evidence that glutamatergic signaling is affected by sleep deprivation and recovery sleep. They support a role for mGluR5 and FMRP in sleep-wake regulation and warrant further studies to investigate their causality and relevance for regulating human sleep in health and disease.

Keywords: PET-MRS imaging; sleep homeostasis; FMRP; BDNF; plasticity

Clinical Trial Registration: www.clinicaltrials.gov (study identifier: NCT03813082)

Statement of Significance

The molecular substrates of increased sleep need and intensity after prolonged wakefulness - referred to as sleep homeostasis - are currently unknown. The glutamatergic system has recently moved to center stage in the search for the molecules underlying sleep homeostasis, yet the evidence is virtually limited to preclinical studies. By combining multi-modal brain imaging (simultaneous proton magnetic resonance spectroscopy and positron emission tomography) and blood sampling, we demonstrate convergent changes in different markers of glutamatergic signaling across prolonged wakefulness and recovery sleep in humans. The findings suggest that glutamatergic signaling in distinct regions of the human brain play an important role in sleep homeostasis and highlight the possible importance of sleep in regulating glutamatergic system activity in health and disease.

Introduction

Sleep has been conserved throughout evolution and is generally assumed to fulfill vital biological functions. This notion is corroborated by the general principle referred to as sleep homeostasis, which assumes that the lack of sleep is predictably compensated by increased sleep need and intensity as reflected by electroencephalographic (EEG) slow-wave activity (SWA; activity in the ~ 0.75-4.5 Hz range) in non-rapid-eye-movement (NREM) sleep.[1] Prevailing current hypotheses posit that sleep homeostasis serves the normalization of synaptic long-term potentiation (LTP) occurring during wakefulness, by synaptic long-term depression (LTD) occurring during NREM sleep.[2–4]

Glutamate (Glu) plays an essential role in the fine-tuned molecular processes underpinning LTP and LTD.[5–7] Overstimulation of metabotropic and ionotropic Glu receptors by excess extracellular Glu is a major culprit of neuronal excitotoxicity and contributes to neuropsychiatric and neurodevelopmental disorders that can be exacerbated by inadequate sleep.[8–10] Suggesting an important contribution of glutamatergic signaling to sleep homeostasis and a role for sleep in keeping extracellular Glu in a homeostatic range, Glu levels in the frontal cortex of freely moving rats rose during prolonged wakefulness and rapid-eye-movement (REM) sleep and decreased during NREM sleep.[11] No comparable data are currently available in humans.

Nevertheless, two key players were recently identified that may orchestrate synaptic plasticity and glutamatergic signaling across the sleep-wake cycle: Homer1a and metabotropic Glu receptors of subtype-5 (mGluR5). Homer1a uncouples mGluR5 from their downstream signaling partners, which leads to synaptic LTD.[12–14] Biochemical, proteomic and imaging studies in mice demonstrated that Homer1a and signaling from group-I mGluRs (mGluR1/5) drive the homeostatic downscaling of excitatory synapses during sleep.[15] In humans, mGluR5 show high expression in brain regions regulating sleep and their functional availability was

increased after prolonged wakefulness.[16] Furthermore, increased mGluR5 availability correlated with behavioral and neurophysiological markers of elevated sleep need, including self-rated sleepiness, unintended sleep during prolonged wakefulness, as well as SWA and slow (< 1 Hz) oscillatory activity in the NREM sleep EEG.[16,17]

Apart from interacting with Homer1a, activation of mGluR5 regulates the expression of fragile X mental retardation protein (FMRP) and brain-derived neurotrophic factor (BDNF), which both play important roles in neuronal plasticity.[5,18–23] Work in Drosophila suggested that the *dFmr1* gene is a molecular regulator of sleep need,[24] and that the expression of FMRP controls sleep time and the sleep loss-induced sleep rebound.[25] Similarly, the expression of BDNF protein in mice has been associated with the rebound in SWA following sleep deprivation.[26] Whereas the effects of prolonged waking on the concentration of FMRP in humans are unknown, for BDNF either an increase or a decrease have been reported.[27,28]

Based upon the evidence outlined above, in this study in healthy human volunteers dynamic changes in brain metabolites, including GLX, Glu and GABA (γ-amino-butyric-acid), were quantified in dorso-lateral prefrontal cortex (dlPFC) and basal ganglia (BG) simultaneously with cerebral mGluR5 availability, as well as FMRP and BDNF levels in blood serum after prolonged wakefulness and following recovery sleep. It was hypothesized that sleep loss increases these potential markers of elevated sleep need and expected that recovery sleep normalizes the waking induced changes. With the exception of BDNF and GABA, all markers quantified revealed sleep loss-induced changes and in part reverted to baseline following recovery sleep, suggesting that glutamatergic signaling involving mGluR5 contributes to the regulation of sleep-wake dependent synaptic plasticity in humans.

Materials and Methods

To visualize the interplay of mGluR5 with its potential molecular signaling partners in sleep-wake regulation, a controlled in-lab study was designed, in which 3-Tesla PET/MR-Spectroscopy scanning and blood sampling were conducted three times, at the same circadian time in baseline, after a night without sleep, and again following recovery sleep. Concentrations of glutamate, the glutamate/glutamine (GLX) ratio and γ-amino-butyric acid (GABA) in basal ganglia (BG) and dorsolateral prefrontal cortex (dlPFC) were measured with dedicated PRESS/MEGAPRESS MRS sequences. The mGluR5 availability was quantified with the novel PET radioligand [^{18}F]PSS232 which is a non-competitive selective antagonist of mGluR5.[29,30] Circulating levels of BDNF and FMRP in human blood were quantified with ELISA.

Study Participants

The study protocol and all experimental procedures were approved by the ethics committee of the Canton of Zürich for research on human subjects. All subjects provided written informed consent prior to the experiments and received financial compensation for their participation, in accordance with the principles in the Declaration of Helsinki.

Thirty-one healthy men completed a within-subject, 1-week sleep deprivation protocol after being screened for medical history and psychological state. All subjects were non-smokers, in good health, had no history of neurologic or psychiatric disease and were instructed not to take any medications or consumed any illicit drugs 2 months prior to the study. Subjects were excluded if they traveled across multiple time zones or performed shift work 3 months prior to study participation. Subjects who prior to the study were not aware of the presence of any sleep-wake disturbances, yet the polysomnographic screening night in the sleep laboratory revealed a sleep efficiency < 75%, sleep apnea or periodic leg movements during sleep

(PLMS) with an index of 5 or more per hour of sleep, were excluded from participation and study enrolment. Table 1 summarizes lifestyle and demographic characteristics of the healthy study sample assessed by validated questionnaires.

Validated German translations and versions of questionnaires were used to assess lifestyle and personality traits. Caffeine consumption was calculated based on the following amounts per serving: coffee: 100 mg; ceylon or green tea: 30 mg; cola drink: 40 mg (2 dL); energy drink: 80 mg (2 dL); chocolate: 50 mg (100 g). Diurnal preference: Horne-Östberg Morningsness-Eveningness Questionnaire;[31] daytime sleepiness: Epworth Sleepiness Scale;[32] depression score: Beck Depression Inventory;[33] personality traits: Eysenck Personality Questionnaire;[34] cognitive assessment: Montreal Cognitive Assessment;[35] trait anxiety: State-Trait Anxiety Inventory;[36] sleep quality: Pittsburgh Sleep Quality Index.[37]

Pre-experimental Procedure and Experimental Protocol

Two weeks prior to the study, participants were required to refrain from all sources of caffeine and wear a wrist activity monitor on the non-dominant arm. During the 5 days prior to the study they were asked to abstain from alcohol intake and to maintain a regular 8-hour night-time sleep schedule, corresponding approximately to the participants' habitual sleep times. Daily log-books and wrist actigraphy verified compliance with the pre-study instructions. Additionally, caffeine and ethanol concentrations in saliva and breath were tested upon entering the laboratory, to confirm participants' abstinence.

Under constant supervision, all subjects completed a within-subject sleep deprivation protocol (Fig. 1), consisting of an 8 hours adaptation and baseline night (time in bed: $11{:}00_{PM}$-$07{:}00_{AM}$), followed by 40 hours of continuous wakefulness, and terminated by a 10-hour recovery night. In baseline (BL), sleep deprivation (TSD) and recovery (RE) conditions,

22 subjects underwent a combined positron emission tomography (PET) with [^{18}F]PSS232 to quantify mGluR5 availability in the brain and proton magnetic resonance spectroscopy (^{1}H-MRS) examination (Division of Nuclear Medicine, University Hospital Zürich). To minimize confounding circadian effects, all measurements were conducted at the same circadian timepoint, starting at $4{:}23_{PM}$ ± 23 min. Due to time and logistic constraints, only two subjects could be PET scanned per experimental week. To optimize data collection, one additional subject was included in each study block (n = 9 in total) as a back-up candidate, participating in the entire experimental protocol, MR imaging and blood sampling, but without [^{18}F]PSS232 injection and PET scanning.

Magnetic Resonance Spectroscopy data acquisition and analysis

The ^{1}H-MRS data were acquired simultaneously with the PET data using a GE 3T combined PET/MR scanner (SIGNA PET/MR; GE Healthcare). Single-voxel edited ^{1}H-MR spectra were acquired from two voxels of interest in the left dorsolateral prefrontal cortex (dlPFC; 30 x 25 x 40 mm^3) and in the basal ganglia (BG; 35 x 30 x 25 mm^3) using the MEshcher-GArwood Point RESolved Spectroscopy (MEGAPRESS) method to quantify GABA as well as Glx and Glu.[38] In addition, a third voxel of interest (VOI) in the BG (25 x 25 x 25 mm^3) was measured with the Point RESolved Spectroscopy (PRESS) method to quantify just Glx and Glu.[38] To ensure a consistent MRS voxel position between subjects, the voxel was carefully positioned based on anatomical landmarks on the T1 image. The T1 weighted MR images were also used to correct for partial volume effects related to the cerebrospinal fluid (CSF) content in the MRS voxel, as well as for gray/white matter correction.

MEGAPRESS: A total of 320 spectra were averaged to obtain the final spectrum. Individual spectra were acquired with a TR of 1800 ms, an echo time of 68 ms, and an eight-step phase

cycle, resulting in a total acquisition time of ~10 minutes. For each metabolite spectrum, 16 water reference lines were also acquired as part of the standard PROBE acquisition.

PRESS: The PRESS spectra were acquired with an echo time (TE) of 35 ms and a repetition time (TR) of 3 ms. 160 spectral averages were acquired to obtain the final spectrum resulting in an acquisition time of 9 min.

Data analysis

The MR spectra were analyzed with LCModel v. 6.3-1,[39] which is a fully automated spectral fitting method. For the MEGAPRESS data, edited spectra were analyzed with a simulated basis set providing metabolite concentrations for glutamine (Gln), glutamate (Glu), glutamate to glutamine (GLX), GABA, N-acetylaspartate, and glutathione. The control parameter sptype = 'megapress-2' was used to avoid mis-assignment of the baseline to GABA. For the PRESS spectra, a standard experimental basis set was used, from which data for creatine, glutamate to glutamine, myo-inositol, N-acetyl-aspartate, and total choline were extracted (Supplementary Figure S1). For all spectra, peaks that were poorly fitted, resulting in Cramer-Rao minimum variance bounds of more than 20% as reported by LCModel or demonstrating apparent artefacts were excluded from further analyses. Specifically, six dlPFC spectra from 5 participants could not be included in the statistical analyses. Moreover, some basal ganglia spectra measured with PRESS were unfortunately automatically overwritten by the scanner software, resulting in missing data points. The numbers of included subjects are indicated throughout the results and in all graphs and/or legends to the Figures.

PET Image Acquisition

A T1-weighted, whole-brain, three-dimensional magnetic resonance (MR) image (resolution: 1 x 1 x 1 mm) was obtained for each subject in parallel to the PET imaging (SIGNA PET/MR 3T whole-body PET/MR unit equipped with an 8-channel head coil; GE Healthcare), to exclude morphological abnormalities and as anatomical standard for the quantification of the PET images. After an automated standard single bolus injection of [^{18}F]PSS232, dynamic PET brain imaging was performed for 60 min. Images were acquired in 3D Mode with Time of flight fully iterative reconstruction (VPFX) using standard MRAC based attenuation correction with a resolution of 1.17 x 1.17 x 2.78 mm^3 and Matrix size of 256 x 256 x 89 voxels binned into 43 timeframes (11 x 1 min, 22 x 2 min, 10 x 1 min). Subjects were instructed to not fall asleep during image acquisition. To verify wakefulness, subjects were instructed to gently press the button of a response box, generating as little movements as possible. As soon as subjects stopped pressing the response box, subjects were alerted via an intercom. Direct contact was avoided, to minimize movement artifacts. Due to technical issues with tracer synthesis, some subjects were not scanned in sleep deprivation and recovery conditions, resulting in missing data points.

Neither injected tracer activity (BL: 164.7 ± 5.2 MBq; TSD: 159.1 ± 3.6.9 MBq; RE: 154.7 ± 3.1 MBq; $p > 0.32$, factor *'condition'*), total activity at the end of synthesis (BL: 2.16 ± 0.10 GBq; TSD: 2.15 ± 0.14 GBq; RE: 1.86 ± 0.12 GBq; $p > 0.23$), nor injected patient dose of [^{18}F]PSS232 (BL: 2.00 ± 0.22 mg; TSD: 1.55 ± 0.17 mg; RE: 1.75 ± 0.16 mg; $p > 0.18$) differed between the conditions.

Image processing and quantification

All processing and quantification analyses were conducted with a dedicated brain PET/MR analysis tool (PNEURO, version 3.7) provided by PMOD Technologies LLC. PET image

processing consisted of within-subject rigid-body motion correction followed by time-series alignment to the MR-T1 image for between scan comparisons. For PET quantification, the T1 image was automatically segmented, separating the MR image into gray matter (GM), white matter (WM) and CSF probability maps. After matching the T1 MR image to the functional PET images, the specific neocortical and subcortical (core brain segments) brain regions were determined using the Hammers-N30R83 brain atlas. Partial volume correction (PVC) was performed automatically in the PNEURO toolbox. A time activity curve (TAC) was calculated for each VOI. Because a single bolus injection was used, the binding potential (BP_{ND}) was quantified with standard SRTM2 [Simplified Reference Tissue Model with fixed k2;[40]] modelling. For modelling, TACs of receptor-rich regions (gray matter VOIs) were compared to the TAC of a receptor-less region (cerebellum) believed mainly to entail non-specific binding.[30]

Assessment of proteins in human serum

Fresh blood was collected immediately before the PET/MRS scans in two 10ml clot activator tubes (BD Vacutainer® CAT). The samples were allowed to clot for about 30 minutes at room temperature (RT) before centrifugation (2.000 relative centrifugal force (RCF) for 10 min). 1.9 mL serum was extracted and purified by a second centrifugation step (12.000 RCF for 5 minutes). The purified serum was aliquoted into multiple 255µ1 samples and stored in Eppendorf tubes (SafeSeal micro tube 1.5 ml, PP, Sarstedt, Nümbrecht) The probes were then snap-frozen in liquid nitrogen and stored at -80°C for future analysis.

Fragile X mental retardation protein (FMRP)

FMRP was studied by a quantitative sandwich enzyme-linked immunosorbent assay (ELISA) purchased prefabricated and ready to use (Human Fragile X mental retardation 1 ELISA kit, MyBioSource, San Diego, California USA). The detection rate of this assay is 15.6 – 1000 pg/ml. A 96-well microplate was pre-coated with a FMRP-specific antibody. Each sample was quantified at least twice for independent confirmation according to the manufacturer's instructions and guidelines (coefficients of inter-assay variation: BN: CV = 14.23 ± 1.52 %, TSD: CV = 11.39 ± 1.68 %, RE: CV = 13.52 ± 1.37 %). The data were normally distributed ($D = 0.08$, $Pr > D > 0.15$, Kolmogorov–Smirnov) and sleep deprivation and recovery sleep did not affect the number of monocytes per ml of blood sample used for FMRP analyses ($p > 0.69$). For technical reasons, some samples could not be reliably quantified and were excluded from the analyses.

Brain-derived neurotrophic factor (BDNF)

Quantification of serum BDNF levels was conducted at the Department of Clinical Psychology and Psychotherapy at the University of Zurich using a 96-Well MULTI-ARRAY® BDNF Assay purchased from Meso-Scale Discovery (MSD®, Rockville, Maryland USA), according to the manufacturer's instructions. No estimate of coefficients of inter-assay variation could be obtained.

Statistical Analyses

All statistical analyses were performed with SAS 9.4 software (SAS Institute, Cary, North Carolina). If not stated otherwise, numbers represent mean ± standard error of the mean (SEM) and the error bars shown in the figures represent the SEM of between-subjects variability. Mixed-effect repeated-measures analyses of variance (ANOVAs) of blood

protein, PET and ^{1}H-MRS data included the within-subjects factor *'condition'* (BL, TSD, RE; supplementary Table S2). The p-values of post-hoc analyses to localize significant differences between the experimental conditions were corrected as follows: Base upon *a priori* hypotheses, the statistical analyses of global mGluR5 availability and FMRP and BDNF levels consisted of three-condition (BL, TSD, RE) Tukey-Kramer correction. The secondary analyses including 81 comparisons (15 pre-selected PET VOIs across 3 conditions and 6 MRS metabolites in 2 brain regions across 3 conditions) were corrected by the Benjamini-Hochberg procedure to reduce the false discovery rate. If not mentioned otherwise, only findings with a corrected p-value below the threshold of $\alpha < 0.05$ were considered significant (supplementary Table S3). Following significant main effects of *'condition'* and post-hoc testing, Mann-Whitney *U* tests of the relative data were performed to illustrate the individual differences in the % change due to the experimental interventions.

Results

Thirty-one healthy men completed this strictly controlled study (Table 1 for demographics; the numbers of study participants contributing to each analysis are specified below). Following 8-hour adaptation and baseline sleep opportunities in the sleep laboratory, all volunteers stayed awake under constant supervision for 40 hours, followed by a 10-hour recovery sleep opportunity. All measurements in BL, TSD and RE started at the same circadian time, at 4:23 pm ± 23 min (Fig. 1). The prolongation of wakefulness increased subjective sleepiness and symptoms of tiredness and lowered the levels of mood and energy. Recovery sleep reversed these changes in subjective state (supplementary Table S1).

Sleep deprivation increases Glu and GLX levels in the basal ganglia

Methodological advances in ^{1}H-MRS have recently permitted the non-invasive detection of naturally occurring changes in tightly regulated metabolite concentrations in circumscribed areas of the human brain. Whereas one recent study suggested that GLX levels in the left parietal lobe decrease over night,[41] previous data from this lab revealed no significant changes after sleep deprivation in GLX/Glu and GABA in the medial prefrontal cortex.[17] Thus, the exact roles in humans of the main excitatory and inhibitory neurotransmitters in circadian and homeostatic sleep-wake regulation remain unclear. Here, the effects of prolonged wakefulness and recovery sleep on the extracellular concentrations of Glu, GLX and GABA in two pre-defined voxels located in cortex (dlPFC) and BG were quantified at the same circadian time in a separate study in 31 newly recruited study participants. Both these regions show pronounced waking-induced changes in mGluR5 availability,[17] and are thought to contribute importantly to sleep homeostasis.[42–44] Consistent with our previous study,[17] sleep deprivation caused no reliable changes in these metabolites in the cortex (Fig. 2, left-hand panel). By contrast, Glu levels in the BG were increased after prolonged waking in 17 of 21 study participants when compared to baseline (Fig. 2, right-hand panel). The mean increase equaled 6.31 ± 2.06 %, which tended to be significant (BL: 1.41 ± 0.02 [arbitrary units]; TSD: 1.50 ± 0.03; TSD *vs.* BL: $p < 0.06$, $n = 21$). Similarly, sleep loss increased the GLX concentration in the BG in 16 of 21 subjects, and the mean increase equaled 9.02 ± 2.53 % (BL: 1.66 ± 0.04; TSD: 1.81 ± 0.05; TSD *vs.* BL: $p < 0.04$, $n = 21$). When relative changes were analyzed, a sleep deprivation-induced increase in both Glu and GLX levels in the BG was confirmed ($p < 0.01$, Mann-Whitney *U* tests). Although both, Glu (TSD: 1.50 ± 0.03; RE: 1.45 ± 0.03; RE *vs.* TSD: 2.8 % reduction) and GLX (TSD: 1.81 ± 0.05; RE: 1.73 ± 0.04; RE *vs.* TSD: 4.2 % reduction) were slightly reduced after recovery sleep when compared to sleep deprivation, these changes did not reach statistical significance.

The levels of GABA remained stable in the BG following sleep deprivation and recovery sleep (BL: 0.45 ± 0.01; TSD: 0.45 ± 0.007; RE: 0.41 ± 0.01; TSD *vs.* BL: $p > 0.8$; RE *vs.* TSD: $p > 0.9$, $n = 21$) (Fig. 2C). Similarly, no significant changes in other metabolites (N-acetylaspartate, glutathione, choline) were detected (supplementary Table S3).

Whole-brain mGluR5 availability is elevated after sleep deprivation and normalized after recovery sleep

To quantify sleep-wake associated changes in the availability of mGluR5 that may occur simultaneously with the above described local changes in Glu, GLX and GABA, the newly developed, highly selective, non-competitive mGluR5 antagonist for PET brain imaging, [^{18}F]PSS232, was employed.[29,30]

When compared to baseline, sleep deprivation induced a consistent increase in whole-brain [^{18}F]PSS232 binding potential reflecting elevated cerebral mGluR5 availability (BL: 1.16 ± 0.04; TSD:1.20 ± 0.04; TSD *vs.* BL: $p < 0.05$, $n = 20$) (Fig. 3). The [^{18}F]PSS232 binding increased from BL to TSD in 15 of 20 subjects in whom PET scans in both conditions were available. On average, the sleep deprivation-induced increase in whole-brain mGluR5 availability equaled 5.53 ± 2.22 %.

To examine whether recovery sleep reverses the wakefulness-induced changes, PET scans were also performed after the recovery night. In 13 of 16 study participants in whom TSD and RE data were available, whole-brain [^{18}F]PSS232 binding was reduced in RE when compared to TSD (TSD: 1.21 ± 0.05; RE: 1.14 ± 0.04; RE *vs.* TSD: $p < 0.01$, $n = 16$). The reduction in mGluR5 availability from TSD to RE equaled 5.77 ± 1.50 %. No difference in [^{18}F]PSS232 binding potential between BL and RE was detected, suggesting that recovery sleep normalized the waking-induced enhancement in mGluR5 availability.

Wake-sleep dependent changes in mGluR5 availability in basal ganglia, amygdala and parietal cortex

Fourteen VOIs previously associated with sleep-wake regulation [16,17,67] were selected for secondary PET image analyses. These VOIs included: caudate nucleus, putamen, ventral striatum, amygdala, dlPFC, orbitofrontal cortex, medial superior frontal cortex, anterior cingulate cortex, parietal cortex, inferior parietal cortex, precuneus, medial temporal lobe, parahippocampal gyrus, hippocampus and insula. Increased [^{18}F]PSS232 binding after prolonged waking was observed in caudate nucleus (BL: 1.15 ± 0.06; TSD: 1.25 ± 0.06; increase: 8.71 ± 4.82 %; TSD *vs.* BL: $p < 0.03$) and parietal cortex (BL: 1.12 ± 0.05; TSD: 1.19 ± 0.05; increase: 6.58 ± 4.46 %; TSD *vs.* BL: $p < 0.03$), and tended to be increased in the amygdala (BL: 1.27 ± 0.07; TSD: 1.38 ± 0.07; increase: 8.66 ± 4.72 %; TSD *vs.* BL: $p < 0.06$; $n = 20$) (supplementary Table S3). When relative changes were analyzed, an increase in mGluR5 availability by sleep deprivation was present in all these three brain regions (Fig. 4, lower panel). Similar to the whole-brain data, recovery sleep normalized mGluR5 availability in caudate nucleus (TSD: 1.25 ± 0.06; RE: 1.14 ± 0.06; reduction: 8.59 ± 3.46 %; RE *vs.* TSD: $p < 0.03$), amygdala (TSD: 1.38 ± 0.07; RE: 1.23 ± 0.07; reduction: 11.31 ± 4.71 %; RE *vs.* TSD: $p < 0.03$) and parietal cortex (TSD: 1.19 ± 0.05; RE: 1.13 ± 0.05; reduction: 5.51 ± 1.95 %; RE *vs.* TSD: $p < 0.03$; $n = 16$) to the level of baseline (RE *vs.* BL: $p_{all} > 0.5$, $n = 16$) (Fig. 4).

Sleep deprivation increases FMRP concentration in blood serum

To tackle the question whether the wake-sleep-related changes in Glu/GLX concentrations and mGluR5 availability in the brain are mimicked by changes in mGluR5-regulated proteins

in peripheral blood, circulating FMRP and BDNF in serum were quantified with enzyme-linked immunosorbent assays (ELISA) in BL, TSD and RE conditions. Intriguingly, prolonged waking increased the blood FMRP concentration in 13 of 23 subjects (BL: 268.52 ± 33.76 pg/ml; TSD: 370.86 ± 31.93 pg/ml; mean increase: 25.86 ± 16.39 %, TSD *vs.* BL: $p < 0.04$, $n = 23$) (Fig. 5). Although the FMRP concentration in RE tended to revert to baseline and the mean FMRP levels in these two conditions did not differ, a difference was neither observed between RE and TSD conditions (RE: 333.89 ± 33.51 pg/ml; RE *vs.* BL: $p > 0.25$, $n = 21$; RE *vs.* TSD: $p > 0.6$, $n = 23$).

In contrast to FMRP, the levels of BDNF were not affected by prolonged waking (supplementary Figure S2).

Discussion

Glutamate is the primary excitatory neurotransmitter of the human brain. Although basic research *in vitro* and in animal models highlights a prominent role for glutamatergic mechanisms in regulating sleep-wake homeostasis,[11,15,17,46–48] knowledge about glutamatergic signaling as a function of waking and sleep in humans is scarce. This study suggests an important relationship between glutamatergic signaling and sleep in humans and supports a role of the basal ganglia in sleep homeostatic mechanisms. More specifically, the data revealed that one night without sleep elicited reliable increases in cerebral Glu/GLX levels and mGluR5 availability, particularly in the basal ganglia, as well as in the concentration of the mGluR5-regulated protein, FMRP, in the blood stream. Given that most of these wakefulness-induced molecular changes tended to normalize after recovery sleep, the findings suggest that sleep may be beneficial to keep glutamatergic signaling in a homeostatic range. In other words, sleep in humans may counteract neuronal dysfunction and

degeneration, which can be caused by excessive glutamate,[8–10] on multiple levels of the metabotropic glutamatergic signaling cascade. Nevertheless, because the concentrations of Glu/GLX and FMRP were not fully restored by recovery sleep, a single recovery night is probably insufficient for the glutamatergic system to fully recover after a night of total sleep deprivation.

Sleep deprivation and recovery sleep induce dynamic changes in basal ganglia glutamate levels

The levels of glutamate in the rat cortical extra-synaptic space rise during waking and decrease during NREM sleep,[11] yet it is currently unknown whether similar changes also occur in the human brain. To examine a glutamatergic contribution to the relief of depressive symptoms after wake therapy, brain levels of Glu, GLX and GABA were previously measured with ^{1}H-MRS in depressed patients undergoing acute and repeated therapeutic sleep deprivation.[49–51] No significant alterations in GLX or its elements were found in different cortical regions (dlPFC, anterior cingulate cortex and parieto-occipital cortex), yet preliminary data indicated that sleep loss increased GLX in subcortical brain regions.[49] Because the baseline levels of GLX and Glu in cerebral cortex differ between depressed patients and healthy controls,[52,53] it is unclear whether these older studies are directly comparable with the present investigation. Nevertheless, previous[17] and current work in healthy controls is consistent with the data in depressed patients.[50,51] It indicates that prolonged wakefulness does not reliably alter the MRS signal compatible with GLX and its constituents in anterior cingulate cortex and dlPFC. It cannot be excluded, however, that the lack of a significant change in GLX in the dlPFC voxel could be related to the voxel composition, which, compared to the basal ganglia voxel was composed of a higher fraction of grey matter.

The data collected in the BG strongly suggest that sleep loss indeed affects glutamatergic signaling on different levels. More specifically, prolonged wakefulness increased Glu, GLX and mGluR5 availability in sub-regions of the basal ganglia, and some of these changes were re-normalized after recovery sleep. The findings corroborate and expand previously published observations from this group, showing that mGluR5 availability was increased after sleep deprivation.[16] The investigation of different brain regions indicated that the basal ganglia are a brain structure that reliably shows sleep-wake related changes in the glutamatergic balance in humans. The dorsal (caudate nucleus and putamen) and ventral (nucleus accumbens and olfactory tubercle) parts of the striatum and the amygdala showed increased mGluR5 availability after sleep loss.[16] Together, the data strengthen the emerging hypothesis that the basal ganglia are a key player in sleep-wake regulation.[54–56] Whereas the observed increase by 5-10 % in Glu levels and mGluR5 availability after extended wakefulness may be considered as small or moderate, the simultaneous changes could mutually amplify each other and cause a substantial increase in glutamatergic signaling after sleep deprivation. Importantly, the present new data demonstrate that recovery sleep is associated with reduced mGluR5 availability, supporting a restorative role for sleep and providing complementary evidence for the mGluR5 signaling cascade to contribute to sleep-wake regulation.

Sleep deprivation impacts on the expression of FMRP

Currently the most specific molecular marker of sleep need is the immediate early gene Homer1a,[46,57] which uncouples mGluR5 from its downstream signaling partners, leading to synaptic long-term depression.[12–15,58] This form of synaptic plasticity may ultimately support sleep dependent recovery processes.[15,59,60] The mGluR5 has been specifically associated with two proteins that may be important for sleep-wake regulation: FMRP and BDNF. Consistent

with experiments in *Drosophila*,[24] the present data reveal elevated FMRP levels after prolonged wakefulness when compared to baseline. A prolonged effect of sleep deprivation, or insufficient recovery sleep, might explain the incomplete normalization in some measurements after recovery. In contrast to the findings *in vivo*, the FMRP concentration in cultured neural cells of sleep deprived rats appeared to decrease with sleep deprivation.[61] Further research is needed to clarify the potential role for FMRP in sleep-wake regulation. Moreover, the concentration of FMRP in human blood serum is low (in the pg range), rendering its quantification difficult, and depends on various possible factors, including genetic.[68] Cautious interpretation and independent replication of this result are, thus, crucial. Similarly, the evidence for a suggested role of BDNF in regulating sleep homeostasis and LTP-like plasticity after sleep deprivation has been equivocal.[28,62] Here, neither sleep deprivation nor recovery sleep revealed consistent effects on BDNF levels in the human serum as quantified with ELISA. The establishment of a reliable method to assess blood serum BDNF still remains a clinical challenge. The discrepancies among the available studies may reflect the methodological difficulties in the reliable quantification of BDNF serum concentration.

Concluding remarks

Although the findings cannot be generalized to female and patient populations because only healthy men were investigated, this study provides convergent evidence that sleep deprivation and recovery sleep affects glutamatergic signaling in distinct regions of the human brain that play an important role in sleep-wake regulation. Nevertheless, the questions remain whether the observed molecular changes regulate the need for sleep or whether they reflect secondary changes associated with the expression of wakefulness and sleep, or both. The present

findings warrant further studies to elucidate the mechanisms that link the homeostatic regulation of sleep and glutamatergic system activity in health and disease.

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Author contributions

Susanne Weigend, Data curation, Formal analysis, Supervision, Investigation, Visualization, Writing-original draft, Writing-review and editing; **Sebastian C Holst**, Data curation, Formal analysis, Supervision, Funding acquisition, Investigation, Visualization, Writing-original draft, Writing-review and editing; **Valerie Treyer**, Resources, Methodology, Supervision, Writing-review and editing; **Ruth L. O'Gorman Tuura**, Software, Methodology, Data curation, Writing-review and editing; **Josefine Meier**, Data curation, Formal analysis, Investigation; **Simon M. Ametamey, Alfred Buck**, Resources, Methodology, Project administration; **Hans-Peter Landolt**, Conceptualization, Resources, Data curation, Supervision, Funding Acquisition, Writing-original draft, Project administration, Writing-review and editing.

Conflict of interest statement

None.

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Table 1. Demographic Data of Study Participants.

Demographic variable	
Age (years)	41.44 ± 20.86
Body Mass Index (kg/m^2)	23.85 ± 2.37
Caffeine Consumption (mg/day)	176.32 ± 144.64
Alcohol Consumption (drinks/Week)	2.98 ± 2.67
Daytime Sleepiness	7.14 ± 3.27
Habitual Sleep Duration (h)	7.44 ± 0.55
Sleep Quality	3.05 ± 1.46
Diurnal Preference	56.00 ± 10.31
Trait Anxiety	29.68 ± 7.55
Eysenck Personality Traits	
Psychoticism	1.95 ± 1.68
Extraversion	7.32 ± 3.40
Neuroticism	2.18 ± 2.68
Lie scale	3.68 ± 2.51
Depression Score	3.45 ± 4.64
Cognitive Assessment	29.14 ± 1.04

Values represent means ± SD (n = 31). Caffeine consumption was calculated based on the following amounts per serving: coffee: 100mg; ceylon or green tea: 30 mg; cola drink: 40 mg (2 dL); energy drink: 80 mg (2 dL); chocolate: 50 mg (100 g). Diurnal preference: Horne-Östberg Morningsness-Eveningness Questionnaire (Horne et al., 1976); daytime sleepiness: Epworth Sleepiness Scale (Bloch et al., 1999); depression score: Beck Depression Inventory (Beck et al., 1961); personality traits: Eysenck Personality Questionnaire (Francis et al., 2006); cognitive assessment: Montreal Cognitive Assessment (Nasreddine, 2005); trait anxiety: State-Trait Anxiety Inventory (Spielberger et al., 1970); sleep quality: Pittsburgh Sleep Quality Index (Buysse et al., 1989).

Figure legends

Figure 1. Experimental protocol. After an adaptation and baseline night, subjects underwent 40hrs of prolonged wakefulness followed by a recovery night. At baseline (BL), after sleep deprivation (TSD), and again after recovery sleep (RE), levels of mGluR5 were measured using positron emission tomography with [^{18}F]PSS232 at the same circadian timepoint (blue dotted lines). Furthermore, distinct brain metabolites were measured with magnetic resonance spectroscopy and blood samples for the quantification of blood BDNF and FMRP levels were drawn at these timepoints. Blue box summarizes type of data collection and number of subjects at the imaging sessions in BL, TSD and RE conditions (blue dotted lines). A cognitive test session was performed every three hours of wakefulness consisting of vigilance (Psychomotor Vigilance Task [PVT]),[63] sleepiness (Karolinska Sleepiness Scale [KSS]),[64] tiredness symptoms (Tiredness Symptoms Scale [TSS])[65] and affective state (Visual Analogue Scales [VAS])[66] testing.

Figure 2: Effects of sleep deprivation and recovery sleep on endogenous brain metabolites in dorsolateral prefrontal cortex (dlPFC, left) and basal ganglia (BG, right). Magnetic resonance spectroscopy yielded levels of glutamate (Glu; A), glutamate/glutamine ratio (Glx; B) and γ-aminobutyric acid (GABA; C) relative to creatine in baseline (BL, dark grey), sleep deprivation (TSD, blue) and recovery (RE, light grey) conditions. Data represent means of arbitrary units (A.U.) ± standard error of the mean (SEM). Numbers on the bars indicate the number of contributing individuals. Black dots represent individual subjects. Missing data points were caused by technical problems during ^{1}H-MRS quantification. Data for Glu and Glx were acquired with PRESS and data for GABA with MEGAPRESS sequences. p-values: Benjamini-Hochberg corrected paired, *t*-tests.

Figure 3: Effects of sleep deprivation and recovery sleep on whole-brain metabotropic glutamate receptor subtype 5 availability (mGluR5). (**A**) Global NonDisplaceable binding

potential (BP_{ND}) after [^{18}F]PSS232 uptake in human brain. Individual data points in baseline (BL, n = 22) and following total sleep deprivation (TSD, n = 20) and recovery sleep (RE, n = 18) are plotted. Connecting lines represent within-subjects changes from BL to TSD and from TSD to RE. The color code identifies individuals exhibiting an increase from BL to TSD (filled black circles) and individuals exhibiting a decrease from BL to TSD (filled red circles); filled grey circles: TSD condition missing. p-values: Tukey-Kramer corrected paired, *t*-tests following significant mixed-model ANOVA with the within-subject factor *'condition'* ($F_{2,36} = 4.52$, $p < 0.02$). (**B**) Box plots of relative changes in global mGluR5 availability from BL to TSD, TSD to RE, and BL to RE. Black dots represent individual subjects. Asterisks indicate significant change scores: * = $p < 0.03$, ** = $p < 0.01$ (Mann-Whitney *U* tests).

Figure 4: Regional differences in the effect of sleep deprivation and recovery sleep on metabotropic glutamate receptor subtype 5 (mGluR5). Upper panel: NonDisplaceable binding potential (BP_{ND}) after [^{18}F]PSS232 uptake in Caudate nucleus (**A**), amygdala (**B**) and parietal cortex (**C**). Individual data points in baseline (BL, n = 22) and following total sleep deprivation (TSD, n = 20) and recovery sleep (RE, n = 18) are plotted. Connecting lines represent within-subjects changes from BL to TSD and from TSD to RE. The color code identifies individuals exhibiting an increase from BL to TSD (filled black circles) and individuals exhibiting a decrease from BL to TSD (filled red circles); filled grey circles: TSD condition missing. p-values: Benjamini-Hochberg corrected paired, *t*-tests following significant mixed-model ANOVA with the within-subject factor *'condition'* (Caudate nucleus: $F_{2,36} = 6.25$, $p < 0.01$; amygdala: $F_{2,36} = 5.54$, $p < 0.01$; parietal cortex: $F_{2,36} = 6.85$, $p < 0.01$). Lower panel: Box plots of relative changes in mGluR5 availability in Caudate nucleus (**A**), amygdala (**B**) and parietal cortex (**C**) from BL to TSD, TSD to RE, and BL to RE. Black dots represent individual subjects. Asterisks indicate significant change scores: * = $p < 0.03$, ** = $p < 0.01$ (Mann-Whitney *U* tests).

Figure 5: Effects of sleep deprivation and recovery sleep on serum fragile X mental retardation protein levels (FMRP). (**A**) Circulating FMRP concentration in human blood serum. Individual data points in baseline (BL, n = 24) and following total sleep deprivation (TSD, n = 27) and recovery sleep (RE, n = 26) are plotted. Connecting lines represent within-subjects changes from BL to TSD and from TSD to RE. The color code identifies individuals exhibiting an increase from BL to TSD (filled black circles) and individuals exhibiting a decrease from BL to TSD (filled red circles); filled grey circles: TSD condition missing. p-values: Tukey-Kramer corrected paired, *t*-tests following significant mixed model ANOVA with the within-subject factor *'condition'* ($F_{2,44} = 3.37$, $p < 0.05$). (**B**) Box plots of relative changes in blood FMRP levels from BL to TSD, TSD to RE, and BL to RE. Black dots represent individual subjects.

List of abbreviations

mGluR5: metabotropic glutamate receptor of subtype 5

Glu: glutamate

GLX: glutamate-to-glutamine ratio

GABA: γ-amino-butyric-acid

BG: basal ganglia

dlPFC: dorsolateral prefrontal cortex

FMRP: fragile-X mental retardation protein

BDNF: brain-derived neurotrophic factor

PET: positron emission tomography

MRS: magnetic resonance spectroscopy

EEG: electroencephalography

SWA: slow-wave activity

NREM sleep: non-rapid-eye-movement sleep

LTP: long-term potentiation

LTD: long-term depression

REM sleep: rapid-eye-movement sleep

PRESS: Point RESolved Spectroscopy

MEGAPRESS: MEshcher-GArwood Point RESolved Spectroscopy

ELISA: enzyme-linked immunosorbent assay

PLMS: periodic leg movements during sleep

SEM: standard error of the mean

VOI: voxel of interest

CSF: cerebrospinal fluid

TR: repetition time

TE: echo time

VPFX: Time of flight fully iterative reconstruction

GM: gray matter

WM: white matter

PVC: partial volume correction

TAC: time activity curve

BP_{ND}: NonDisplaceable binding potential

SRTM2: Simplified Reference Tissue Model with fixed k2

RT: room temperature

RCF: relative centrifugal force

FDR: false discovery rate

BL: baseline

TSD: total sleep deprivation

RE: recovery

PVT: psychomotor vigilance task

KSS: Karolinska sleepiness scale

TSS: tiredness symptoms scale

VAS: visual analogue scale

ANOVA: Analysis of variance

denDF: denominator degrees of freedom

numDF: numerator degrees of freedom

Figure 1

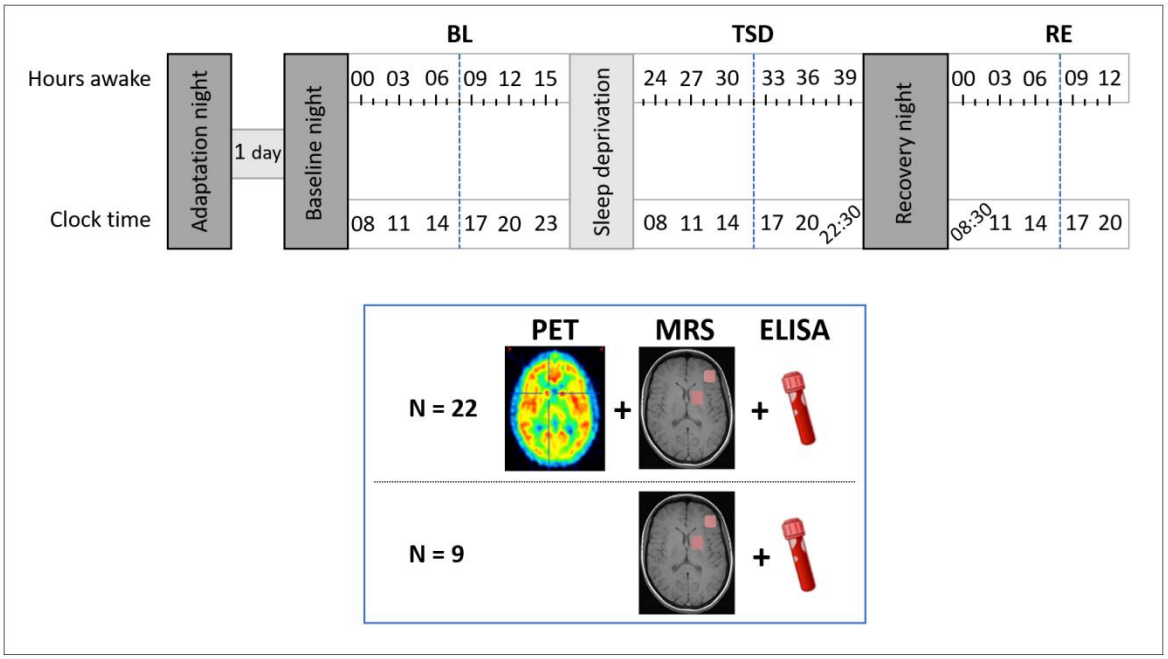

Figure 2

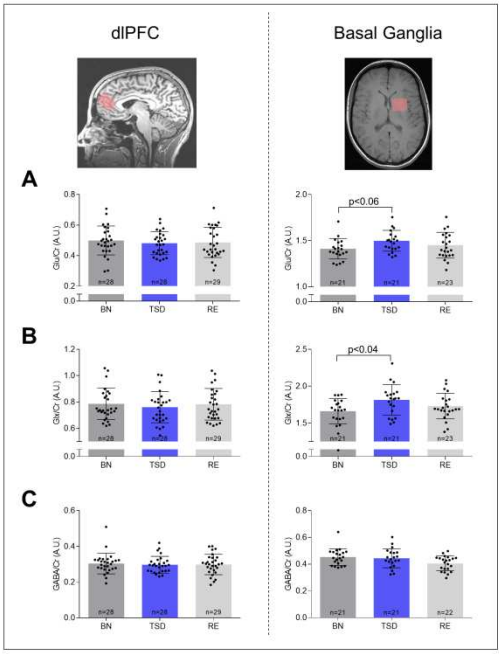

Figure 3

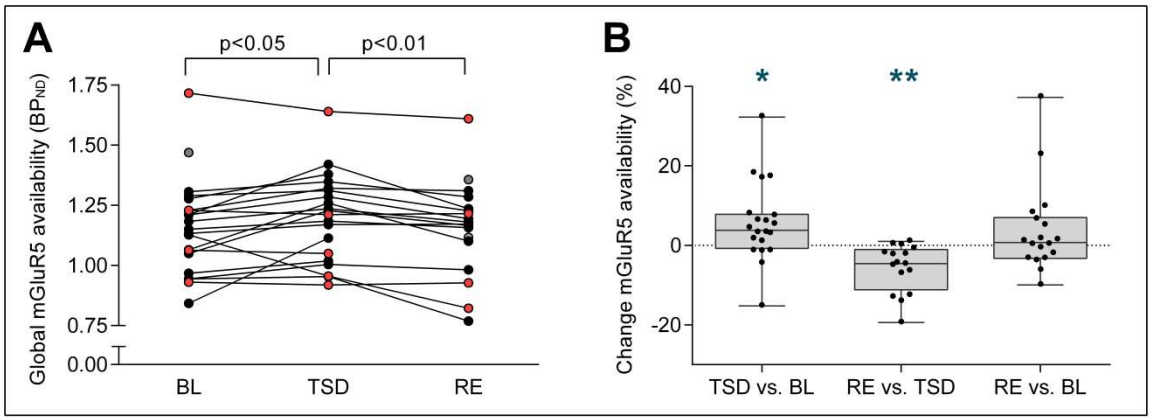

Figure 4

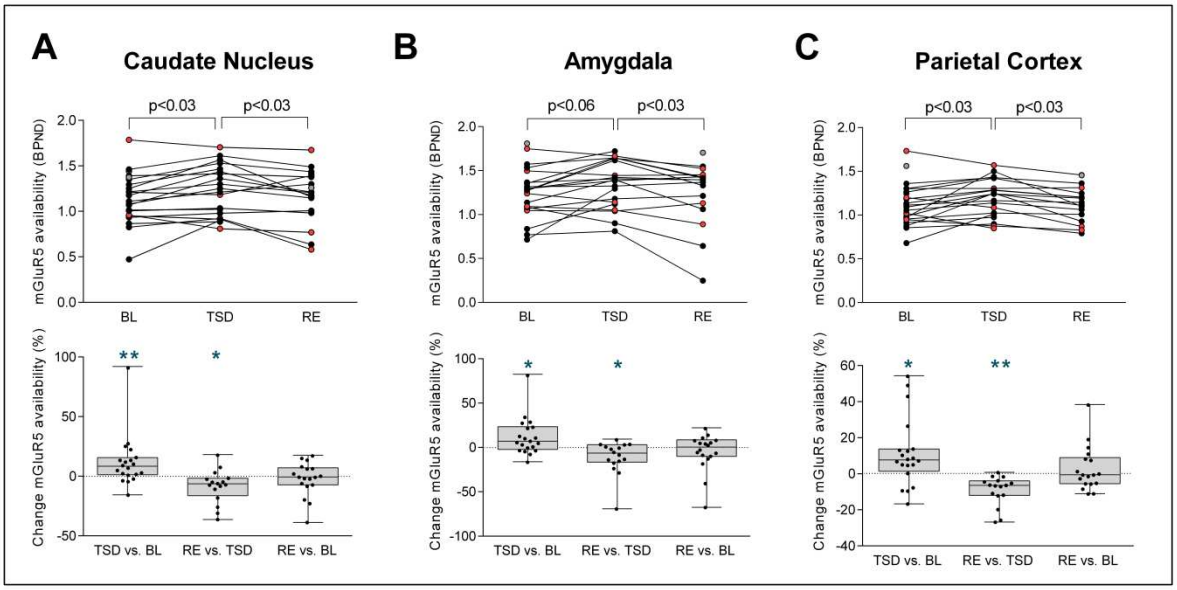

Figure 5